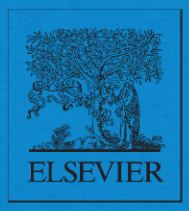

Reprinted from
Volume 141, 12 February 2015

Separation and Purification Technology

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On-line Access via: www.elsevier.com/locate/seppur

Separation and Purification Technology 141 (2015) 269–275

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Complete clarification solution for processing high density cell culture harvests

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ARTICLE INFO

Article history:
Received 29 August 2014
Received in revised form 3 November 2014
Accepted 3 December 2014
Available online 15 December 2014

Keywords:
Clarification
Depth filtration
Flocculation
Separation
pDADMAC
Purification

ABSTRACT

Traditional clarification of mammalian cell culture typically combines multiple separation technologies to remove cells in a primary step, and colloids in a secondary step. Recent advances in mammalian expression systems have not only led to cell cultures with very high density and increased protein titers, but also to feed streams with a high proportion of solids and colloids that reduce the efficiency of the separation step. Issues with scale-up, flexibility and installation limit the attractiveness of centrifugation, whereas clarification solely by depth filtration can be costly due to high filter areas required. Therefore, an efficient manufacture process must be developed to overcome these restrictions. In this study we present a complete separation solution that combines cell harvest pretreatment with a polycationic flocculating agent (polydiallyldimethylammonium chloride or pDADMAC), followed by depth filtration using depth filters that were specifically developed for filtration of flocculated or precipitated feed streams. Multiple antibody feed streams treated with pDADMAC and subsequently filtered using pretreatment adapted depth filters resulted in improved removal of cells and colloids, increased clarification throughput, efficient reduction of DNA and high process yield. The residual level of pDADMAC in the filtrates was assessed by surface plasmon resonance spectroscopy. Overall, this next generation clarification solution presented herein led to a robust, high yield, economical separation process with enhanced impurity removal that can be readily incorporated into current clarification platforms.

1. Introduction

Downstream purification of antibodies requires the clarification of harvested cell culture broth to remove plugging components and enable economic reuse of chromatography columns for many cycles. Traditional clarification of mammalian cell culture harvests consists of one or a combination of several separation technologies, such as centrifugation, tangential flow filtration (TFF) and normal flow filtration (NFF).

In order to increase the productivity, intense efforts in mammalian expression systems have been invested. While in the past decades cell culture processes of monoclonal antibodies resulted in titers of about 1 g/L, productivities exceeding 10 g/L are now reported [1]. This is generally combined with cell density levels of >20 million cells/ml and cell viability <50% [2,3]. In such harvests the high proportion of solid biomass (cells, cell debris and colloids) and high content of soluble byproducts (host cell proteins and DNA) reduces the performance of traditional separation technologies. Disk stack centrifuges require greater discharge frequency and/or acceleration rates and produce larger pellets, potentially leading to yield issues. In addition, the rise in colloid loads with high biomass and low cell viability harvests result in early plugging of the subsequent depth filter [4]. Likewise, open channel TFF cassettes exhibit lower flux and capacity due to higher solid content, hence requiring higher filtration area. NFF filters require more internal volume to hold the increased biomass and may experience earlier breakthrough of fine particulates. This leads to a lower capacity of the bioburden filter or the need to implement a second depth filter device.

To overcome these limits of traditional separation technologies, various pretreatment approaches based on precipitation or flocculation techniques have been developed and evaluated over the years [5]. While precipitation relies on lowering the solubility of

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http://dx.doi.org/10.1016/j.seppur.2014.12.002
1383-5866/

solutes in order to create solid particles, flocculation relies on the aggregation of particles. Flocculation occurs if the van-der-Waals attraction between particles outweighs the electrostatic repulsion according to DLVO theory (Derjaguin, Landau, Verwey and Overbeek). Flocculating agents can be used to trigger the aggregation of dispersed particulates into larger-size clusters. Typically, positively charged polymers are used as flocculating agents (e.g. polyamines, polyethylenimine, polydiallyldimethylammonium chloride (pDADMAC, Fig. 1) or chitosan). The flocculation mechanism using polycationic polyelectrolytes has been extensively studied [6,7]. They are widely used in wastewater treatment and paper manufacturing, but recent studies have looked at applying flocculation in biopharmaceutical applications [8,9]. However, a settling or centrifugation step is often used in order to separate flocculated material, because traditional depth filters cannot handle high solid feed streams.

The objective of this study was to develop an efficient separation solution that comprises cell harvest flocculation, followed by a depth filtration. Multiple antibody cell culture fluids were treated with pDADMAC and filtered directly through pretreatment adapted depth filters, without any additional adjustment step. The separation effectiveness for pDADMAC-mediated flocs was benchmarked with the performance of a traditional depth filtration approach. In addition, pDADMAC dose–effect on harvest particle size distribution was assessed to provide a better understanding of the pDADMAC flocculation mechanism.

2. Materials and methods

2.1. Cell culture and protein expression

Antibodies used in this study were produced in Chinese hamster ovary (CHO) cell lines, which reached a cell density of 1.3–1.6 $\times$ 10^7 total cells/mL, turbidity of >1000 NTU (a 1:10 dilution with water gave for mAb1 1830 NTU and for mAb2 1880 NTU) and viability at harvest of 37% and 70% for mAb1 and mAb2, respectively. mAb1 titer was 3.0 g/L, whereas mAb2 was expressed at the level of 1.5 g/L. The monoclonal antibody mAb3 feed stream (1.2 $\times$ 10^7 total cells/mL with 80% viability and turbidity >1000 NTU) was solely treated with pDADMAC in order to analyze the particle size distribution upon flocculation.

2.2. Flocculation of cell culture

The optimum dose of pDADMAC (obtained as purified 10% solution from Merck KGaA, Darmstadt, Germany) was first defined in a screening experiment where 40 mL of the representative cell culture harvest were mixed with increasing amounts of the polycationic polymer ranging from 0 to 0.2% wt%. After incubation for 15 min at room temperature, the flocculated cell culture was centrifuged at 2000$\times g$ for 5–10 min (Hereaus Instruments, Hanau, Germany), followed by filtration using 0.2 μm Millex® filters. Centrate turbidity of the various samples was determined using a portable turbidimeter model 2100P ISO (Hach, Berlin, Germany). Filtered supernatants were submitted for product concentration, HCP and DNA analysis and compared to non-treated, centrifuged feed.

2.3. Filtration experiments

The optimized flocculation condition, which was judged mainly by means of centrate turbidity, was applied on a larger volume of feed. A portion of the cell culture harvest of about 4 L was treated with pDADMAC and clarified using pretreatment adapted depth filters (Clarisolve® 20MS, 40MS and 60HX graded depth filters (all μPOD device format of 23 cm^2, Merck KGaA, Darmstadt, Germany)). 20MS is designed to clarify smaller aggregated particles (of 20 μm), 40MS for medium aggregated particles (of 40 μm) and 60HX for larger aggregated particles (of 60 μm). In parallel, the untreated cell culture harvest was filtered using traditional depth filters featuring diatomaceous earth layers of different pore size (Millistak D0HC and X0HC depth filters, Merck KGaA, Darmstadt, Germany). The traditional depth filters were assembled either inline at a 1:1 filter area ratio or decoupled using the labscale POD format of 270 cm^2 for D0HC. For the various filtrates, the capacity of each depth filter grade was evaluated using the P_{max} constant flow sizing method [10]. Briefly, the feed solution was pumped through the various test depth filters at a constant filtrate flux of 95–138 L/(m^2h). The differential pressure, cumulative filtrate volume and turbidity were recorded as a function of process time. The depth filtrate quality was assessed by measuring the capacity of a sterilizing grade filter (0.5/0.2 μm Millipore Express® SHC, 3.5 cm^2 filter area, Merck KGaA, Darmstadt, Germany) at constant pressure of 5 psi (0.34 bar). The sizing of these filters was performed according to the flow decay V_{max} method [11].

2.4. Analytical assays

The turbidity was measured using the 2100P ISO portable turbidimeter (Hach, Berlin, Germany). The product concentration was measured with an IgG assay utilizing the COBAS INTEGRA 400 plus (COBAS® INTEGRA400 plus, Roche Diagnostics, Germany). CHO-Host Cell Protein (HCP) levels were assessed using a set of anti-CHOP reagents, including CHO HCP standard and proprietary anti-CHO HCP antibodies. Analysis was performed applying ELISA-based assay. The HCP clearance was calculated as logarithmic removal factor (LRV):

$$\mathrm{LRV} = \log \frac{\text{ng HCP/ml feed}}{\text{ng HCP/ml filtrate}} \quad (1)$$

Residual host cell DNA was detected by quantitative PCR (qPCR). Similarly, the DNA clearance was calculated using the following equation:

$$\mathrm{LRV} = \log \frac{\text{pg DNA/ml feed}}{\text{pg DNA/ml filtrate}} \quad (2)$$

The particle size distribution of untreated and flocculated feed expressing mAb3 was measured using a coulter counter (CASY Model TTC analyzer, Roche Diagnostics GmbH) which has a detection range from 3.2 to 120 μm. The amount of residual pDADMAC was determined by surface plasmon resonance technology (SPR) using the Biacore T200 enhanced sensitivity (GE Healthcare) according to polymer's manufacture. The samples obtained after filtration were diluted 1:10 before being subjected to SPR. The initial binding rates were plotted against the pDADMAC concentration resulting into a regression curve with a correlation coefficient R^2 = 0.981.

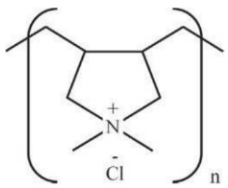

Fig. 1. Chemical formula of pDADMAC where the positively charged quaternary ammonium group in the heterocyclic aromatic ring is denoted.

3. Results and discussion

3.1. Flocculation optimization study

In order to characterize the effect of the polymer concentration, cell culture fluids containing monoclonal antibody mAb1 or mAb2 were mixed with increasing amounts of pDADMAC. For either feed stream a decrease in turbidity was observed as compared to that of the untreated feed stream (Fig. 2). This indicates that pDADMAC is effective in flocculating cells and cellular debris. The polycationic polymer presumably adsorbs on the surface of negatively charged particles by electrostatic interaction. Thereby it induces the formation of larger-size particles that sediment faster by applying centrifugal force at low accelerations. At higher polymer doses the turbidity of the centrate increases again. This turbidity increase is not caused by the polymer alone as it shows no turbidity increase over concentration range used (data not shown), and the acceleration rate was relatively low to have a significant impact on the flocs, but cannot be completely excluded. It seems that the turbidity is rather affected by floc properties. It is assumed that the flocculant dosage depends on the cell density, as the portion of total biomass (cells, colloids) increases with increasing cell density. Turbidity is significantly reduced over a wide range of pDADMAC concentrations indicating that one can have a robust process from batch-to-batch. The lowest centrate turbidity was achieved with 0.05% wt% pDADMAC. This final concentration was taken as the appropriate polymer dosage for the clarification of mAb1 and mAb2.

No product loss was determined for all flocculant conditions tested, implying the high specificity of pDADMAC for negatively charged particles. The protein concentration analysis of filtered supernatants revealed recoveries of >98% compared to untreated feed streams. An unusual increase of product recovery was observed following pDADMAC flocculation with more than 0.05% wt%. This may be due to interference from relatively higher level of residual polymers in these samples impacting the reliability of the IgG quantification assay. This effect has not been observed when the samples were measured with an analytical Protein A chromatography method (data not shown).

3.2. Effect of the polymer on the particle size distribution

The particle size distribution of the cell culture harvest before and after flocculation was investigated. As the feeds streams containing mAb1 or mAb2 were not analyzed for particle sizes, an aliquot of mAb3 harvested cell culture fluid was submitted for particle size analysis (Fig. 3). The mean particle diameter in the untreated cell culture suspension was determined to particle size of about 22 μm. It is assumed that a similar behavior for mAb1 and mAb2 expressing cell cultures would be obtained [12]. Upon a cascade of electrostatic interactions, the particle size distribution is shifted towards larger and likely denser flocs. Interestingly, the higher dose of pDADMAC has not resulted in the formation of bigger particles (>22 μm) or higher volume of the particles with a mean diameter of 22 μm. The analysis rather revealed that the average size decreased (to about 20 μm) with the highest dose of pDADMAC used. Furthermore, the volume of the particles in the range of 10 μm ("unflocculated peak") increased with pDADMAC concentration. This all supports the hypothesis that a generous polymer addition destabilizes larger size clusters and correlates with the increase in centrate turbidity upon flocculation of high polymer dose.

The second, smaller particle size peak in the untreated feed stream represents a population that might be hard to remove even with traditional separation techniques. Kempken et al. [13] assessed the clarification efficiency of mammalian cell cultures by using a disk stack centrifuge and determined that a substantial colloid load below 5 μm was present in the liquid phase after cell separation. Notably, there is a decrease in volume of particles with a mean diameter of 4–5 μm. This population was likely stabilized in pDADMAC-mediated flocculates.

3.3. Filter capacity throughput and protein recovery

A depth filtration train consisting of D0HC followed by X0HC is targeted for traditional clarification of biopharmaceuticals produced in mammalian cell culture. The summary of filtration performance results on mAb1 and mAb2 feed streams is shown in Table 1. The endpoint throughputs and pressure drop values are reported, in most cases filtration experiments were run until a pressure drop of >15 psi was reached. The filtration train consisting of D0HC/X0HC in series at a 1:1 filter area ratio was evaluated for untreated broths resulting in depth filter loadings of 118 and 112 L/m^2 for mAb1 and mAb2, respectively. The higher endpoint pressure for D0HC in this two-stage depth filtration set-up indicates that this grade predominately contributes to the cell and colloid retention. The very low turbidity of final filtrates compared to that of bulk harvests (>1000 NTU) implies very good retention by

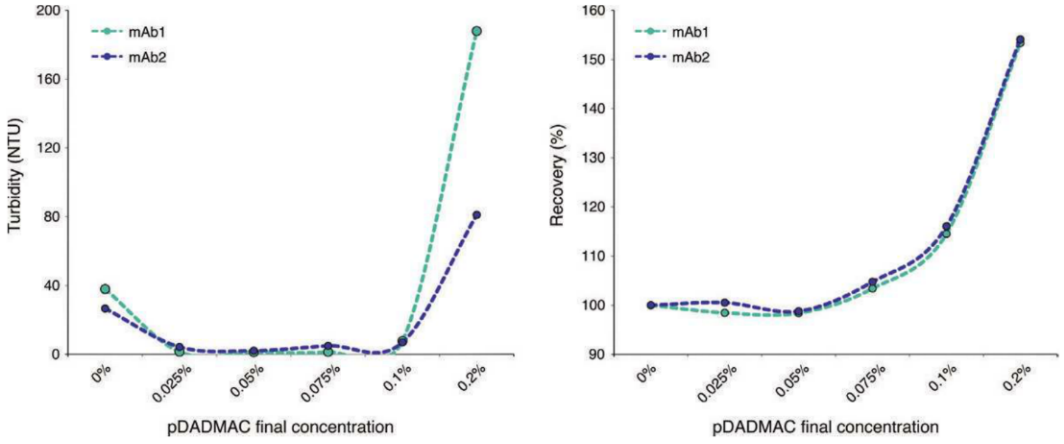

Fig. 2. Flocculation optimization study with increasing pDADMAC amounts and post centrifugation. Turbidity (left) and recovery (right) profiles are shown as a function of pDADMAC concentration for mAb1 (dark blue) and mAb2 (light blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

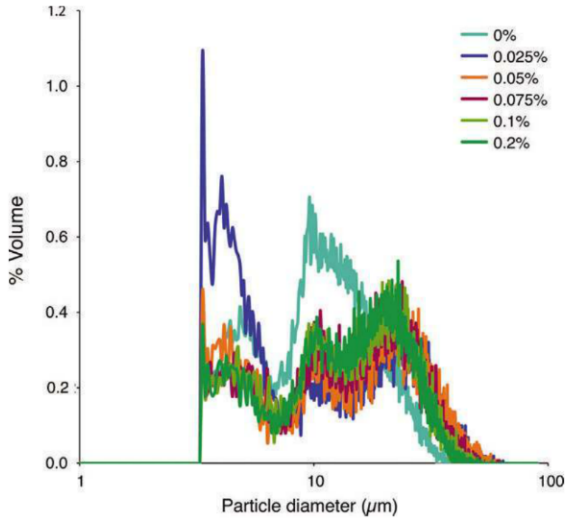

Fig. 3. Particle size distribution of representative CHO cell culture feed stream (mAb3) that was flocculated and submitted for analysis. Data are shown for untreated (light blue) and pDADMAC-treated cell culture feed stream. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the filter media. The throughput results obtained from the uncoupled assembly suggest that the capacity of the secondary filter may exceed 500 L/m^2 and a ratio of 3:1 or 4:1 (D0HC to X0HC) may be more adapted.

In comparison, the experimental assays performed on the bulk harvest that was flocculated with a pDADMAC dose of 0.05% wt% and subsequently filtered through pretreatment adapted depth filters provided promising capacities. All pretreatment adapted depth filters demonstrated trial capacities of >350 L/m^2 while the pool turbidity was similar to that of the control run (untreated feed processed using D0HC/X0HC filtration train). No increase in the turbidity of all filtrates was observed 12 h post depth filtration (data not shown), implying that flocculation was complete at the time of filtration. 20MS and 40MS filters performed with higher capacities compared to 60HX, the later is designed to retain particles of pretreated feed streams of 60 μm. Therefore, data suggest that the retention was not solely achieved by sieving, but adsorption may also contribute to it. Furthermore, the depth filters are nominally rated and process conditions could have an effect on the retention efficiency of the filters. There was little difference in depth filtration performance of 20MS and 40MS for mAb1, with a 10% higher 40MS filter capacity compared to 20MS for mAb2.

Among other factors, the recovery of antibodies was set as criterion for a successful clarification. The analysis revealed that >97% and >92% of mAb1 and mAb2, respectively, were recovered

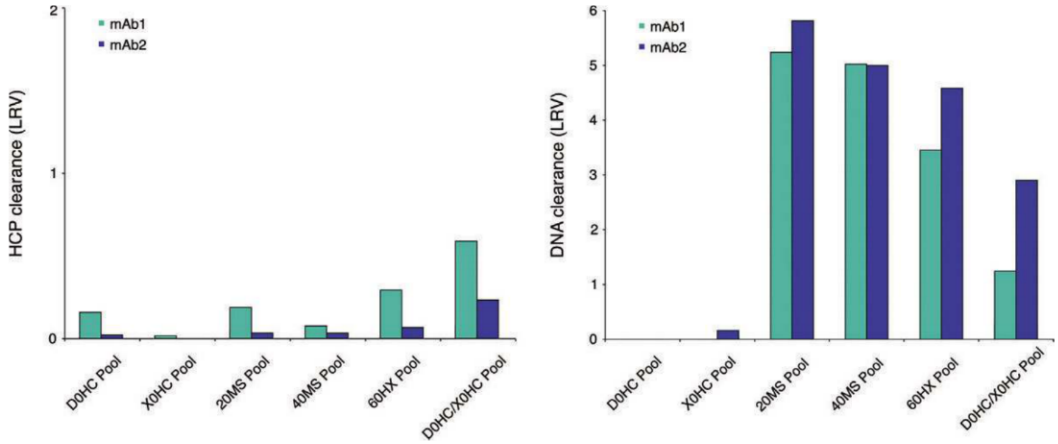

Fig. 4. Process performance in terms of soluble impurity level. HCP (left) and DNA (right) clearance shown in LRV (log reduction value) for mAb1 (light blue) and mAb2 (dark blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Filtration performance obtained for depth and sterile filters in the clarification of monoclonal antibodies mAb1 and mAb2 using either the traditional ("untreated") or the pDADMAC flocculation approach.

Protein	Flocculation condition	Depth filter	max Δp (psi)	Throughput (L/m^2)	Pool turbidity (NTU)	Protein recovery (%)	Sterile filter throughput (L/m^2)
mAb1	Untreated	D0HC/X0HC	27/3[a]	118	1.1	82	>10,000
	Untreated	D0HC	24	119	28.6	97	154
	Untreated	X0HC	3.5	489	4.4	95	>10,000
	0.05% pDADMAC	20MS	15	504	2.5	98	>10,000
	0.05% pDADMAC	40MS	25	501	2.6	97	7550
	0.05% pDADMAC	60HX	26	359	3.1	95	4661
mAb2	Untreated	D0HC/X0HC	27/3[a]	112	2.0	93	>10,000
	Untreated	D0HC	14	109	53.4	93	47
	Untreated	X0HC	5	486	3.8	93	6217
	0.05% pDADMAC	20MS	19.5	450	3.1	92	2299
	0.05% pDADMAC	40MS	21	498	2.9	93	>10,000
	0.05% pDADMAC	60HX	19	358	4.0	89	900

[a] Cumulative endpoint pressure.

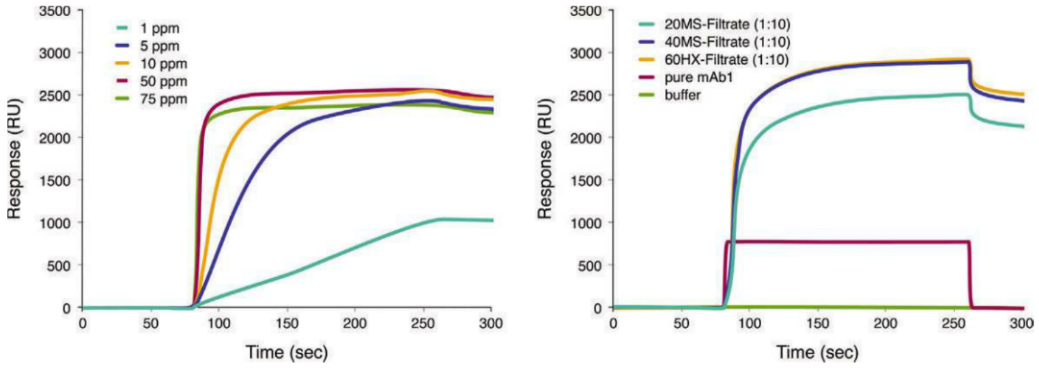

Fig. 5. Residual pDADMAC detection using SPR spectroscopy. Response levels of pDADMAC in standards (left) and samples (right) are shown in sensograms, where the samples obtained after depth filtration were diluted 1:10 before being injected. Running buffer: PBS.

in the filtrates compared to untreated feed streams. Thus, flocculation with pDADMAC and subsequent filtration through pretreatment adapted depth filters were efficient in clarifying mAb1 and mAb2.

The sterile filter capacities for each filtrate are also shown in Table 1. Filtrates denoted with V_{max} values >10,000 L/m^2 did not show significant flux decay within the experimental range and hence are considered to be non-plugging fluids.

In summary, the flocculation of the cell culture harvest with pDADMAC and subsequent filtration using pretreatment adapted depth filtration simplifies the clarification of mammalian cell culture broths expressing monoclonal antibodies compared to depth filtration without prior flocculation. The clarification throughput could be increased versus the standard clarification train by filtering flocculated broths through pretreatment adapted depth filters designed to capture larger particles. Throughput is likely dependent on the total cell density and viability. Nevertheless, the use of a flocculant in combination with pretreatment adapted depth filtration provides robust performance with the potential of reducing clarification to a one-stage filtration process. Other pretreatment approaches showed comparable simplification [12,14]. This compressed and simplified process can be applied to various mAb-containing cell culture harvests with effective removal of cells and cell debris.

3.4. Removal of soluble impurities from mAb feed streams

The clarification experiments with mAb1 and mAb2 demonstrated an efficient removal of cells and cell debris. At this stage, any additional clearance of soluble contaminants (such as host cell DNA and proteins) will likely have positive impact on the downstream unit operations, although purification performance might be culture dependent [15]. The removal of host cell proteins (HCP) and nucleic acids (DNA) during the clarification process of mAb1 and mAb2 has been investigated in this study and compared to the corresponding untreated cell culture harvest Fig. 4. Following filtration of the untreated cell culture harvest with D0HC/X0HC, the level of HCP remains high compared to the feed material, although for some harvests a more effective HCP clearance is seen. The removal of HCP could not be significantly improved by flocculation and use of pretreatment adapted depth filters. The DNA level was not significantly reduced when the untreated feed streams were filtered through D0HC/X0HC depth filters. As the residence times of the harvests within the depth filters were similar for coupled and decoupled assemblies, the 4-fold higher loadings on X0HC in the decoupled mode obviously lowered DNA clearance in the secondary filtrate pool. By contrast, mAb1 and mAb2 flocculated feed streams that were processed using pretreatment adapted depth filters demonstrated >3 $\log_{10}$ and >5 $\log_{10}$ clearance of (strongly negatively charged) DNA. 20MS and 40MS filters showed highest removal of host cell DNA greater than 5 logs. These results demonstrate good host cell DNA clearance as a result of the flocculation process with pDADMAC.

3.5. Detection of residual flocculant

As polycationic polymers are becoming more and more attractive in biopharmaceutical applications, they have been investigated for their cytotoxicity to minimize the risk of any harm for patients [16,17]. Previous reports based on *in vitro* assays have shown that polycationic polymers can change cell morphology and induce hemolysis depending on the polymer property, dose and incubation time. Polyethylenimine is the most cytotoxic polymer tested, whereas the results on pDADMAC-mediated hemolysis suggest that up to a dose of 10 ppm no disturbance of the red blood cell membranes was observed [16].

In the present study, we have analyzed for residual pDADMAC after depth and sterile filtration applying SPR spectroscopy. The sensograms of pure pDADMAC-standards and pDADMAC-containing samples clarifying mAb1 are shown in Fig. 5. The response signal of standards increased with increasing pDADMAC

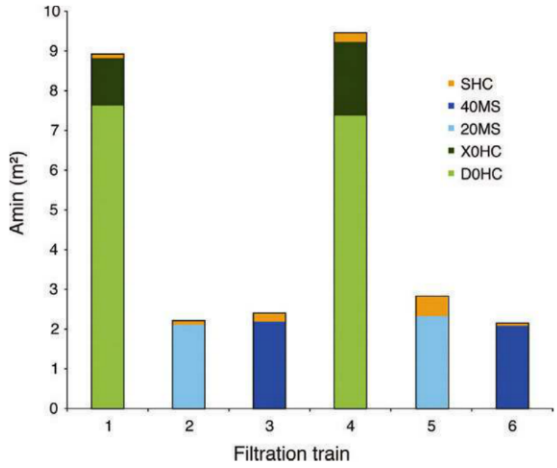

Fig. 6. Calculation of the minimal filter area (A_{min}) for depth (D0HC, X0HC, 20MS and 40MS) and sterile (SHC) filters. Filter requirements needed to process a 1000-liter batch are depicted in this figure.

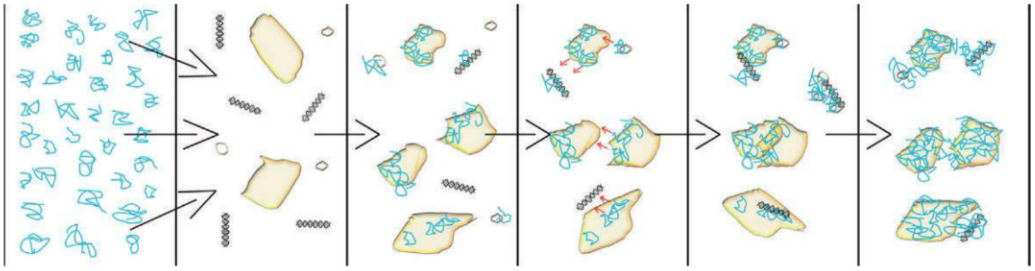

Fig. 7. The electrostatic patch flocculation mechanism by pDADMAC. Negatively charged particles such as cells (big particle), nucleic acids (helix) and most CHO proteins (white particle) are flocculated by increased amounts of polycationic pDADMAC (blue) in a 'patchwise' manner, where patches of excess positive charge on one particle surface adsorb onto the negative parts of an oppositely-charged particle until the particles get destabilized at higher levels of polymer surface coverage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentration, indicating a concentration-dependent detection assay that can be applied to measure pDADMAC up to 1 ppm. Significant binding rates were obtained for filtrates, whereas pure mAb1 (without dilution) and running buffer resulted in no or low response signal. The control sample of the bulk harvest without addition of pDADMAC would have provided the background response signal, which unfortunately has not been included in this study. Therefore, absolute values of pDADMAC clearance were not calculated, but will be shown for another biomolecule elsewhere, where the background coming from the bulk harvest will be subtracted. It seems that for mAb1 less pDADMAC remained in the treated sample processed through 20MS than through 40MS depth filter, implying a higher usage and removal of pDADMAC in the former case. This needs to be verified, however, lower residual pDADMAC amount is in correlation with V_{max} values that showed a cleaner filtrate observed upon a filtration through 20MS. It is expected that cation exchange steps, typically found in most mAb processes, would reduce pDADMAC concentrations.

3.6. Filter area determination

To determine the filter area needed for a 1000 L harvest processed in 3 h, different mathematical models for the plugging process can be applied [18]. For the tested streams the empirical polynominal model was used. In Fig. 6 relevant filtration configurations are shown with filtration trains 1–3 for mAb1 and 4–6 for mAb2. For both streams a total minimum filter area (A_{min}) of about 9 m^2 for D0HC and X0HC is required. It is expected that for clarifying other antibodies expressed in similar cell lines and in similar media as the ones used in this study, comparable filter areas will be needed for a robust and generic filtration train. Pretreatment and subsequent filtration using the pretreatment adapted depth filters results in a significant reduction of minimal filter area (train 2 and 3 for mAb1, train 5 and 6 for mAb2). Other benefits are the higher dirt holding capacity of those filters compared to the use of conventional depth filters and lower flush volumes required [12].

4. Conclusion

In this study we presented a novel process for harvesting high density mammalian cell culture supernatants. Given that cells, nucleic acids and most host cell proteins of a CHO cell culture are usually negatively charged at neutral pH, flocculation by a polycationic polymer is promising. The adhesion of pDADMAC to negatively-charged particles leads to the formation of larger-size clusters which are easily separated in a solid–liquid separation process. At higher polymer levels, flocs are destabilized by charged and/or steric repulsive interaction of polymer chains that results in later turbidity increase and change in the size distribution Fig. 7.

Filtration throughput of about 500 L/m^2 was observed by filtering pDADMAC-flocculated broths with pretreatment adapted depth filters. This leads to the reduction of minimal filter area by more than 4-fold compared to the 'classical' two-stage depth filtration. Furthermore, the clarified cell culture harvest could be led directly from primary clarification to bioburden filtration, thus enhancing the efficiency of the unit operation. The dosage of pDADMAC should be optimized with each feed stream. However, it appears that a polymer quantity of <30 pg/cell seems to be appropriate (0.048% wt% for 16 million cells/ml). To address pDADMAC residual level post clarification, a reliable SPR based detection method has been demonstrated. In conclusion, the flocculation with pDADMAC and subsequent filtration through pretreatment adapted depth filters could improve and simplify the clarification process of challenging high cell density and low viability mammalian cell culture harvest applications.

Acknowledgement

We would like to thank Dipl.-Ing. (FH) Gabriele Neumann, Susanne Rahnfeld and Angelika Strauch for their excellent technical contributions on this project. We sincerely thank Dr. Herb Lutz, Dr. Nripen Singh and Dr. Helge Berg for critical reading of the manuscript.

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